Isolation and Characterization of Antibiotics Resistant Bacteria from Raw Milk

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Abstract—Objective: Isolation and identification of antibiotics resistant bacteria in raw milk. Methods: Luria-Bertani plates containing ampicillin (32 μg/mL), clindamycin (4 μg/mL), or chloramphenicol (32 μg/mL) were used in isolating the antibiotics resistant bacteria from samples; 16S rRNA analysis method was used to identify the isolates; blood agar plates were used in hemolysis assay; K-B disk diffusion method was used to confirm the resistant phenotypes. Results: 60 raw milk samples collected from Zhangjiakou were screened for antibiotic resistant bacteria. The screening results showed that 48 (80%) samples had isolates resistant to more than one antibiotic, 39 (65%) samples had isolates resistant to more than two antibiotics, and 14 (23.33%) samples had isolates resistant to three antibiotics tested. Totally, 48 strains were isolated resistant to ampicillin, 46 strains were isolated resistant to clindamycin, and 21 strains were isolated resistant to chloramphenicol. Ten strains (resistant to three antibiotics) were randomly selected for 16S rRNA analysis and they were identified as Serratia marcescens (1), Pseudomonas aeruginosa (8), and Enterobacter cloacae (1), respectively. Hemolysis test results showed those 2 strains with α-hemolytic phenotype, 2 strains with β-hemolytic phenotype, and 6 strains with γ-hemolytic phenotype. Furthermore, with K-B disk diffusion method, 10 isolates were validated resistant to 3 antibiotics tested except that one isolate intermediated to chloramphenicol and two isolates susceptible to chloramphenicol. Conclusion: Raw milk is the reservoir of a variety of antibiotics resistant bacteria; antibiotic plates can be used for preliminary screening of antibiotics resistant bacteria from raw milk samples.

Keywords—milk; resistant bacteria; isolation and identification; K-B disk diffusion method

I. INTRODUCTION

A variety of bacteria can be separated in raw milk, including Lactobacillus, Pseudomonas, Micrococcus, Bacillus, Clostridium, Listeria, Enterobacteriaceae, and etc.1 Some bacteria are important cattle pathogens responsible for mild or severe mastitis. Among them, Staphylococcus aureus is the most common pathogen isolated from 25-50% mastitis cases.3 Other pathogens, such as Streptococcus agalactiae, Streptococcus uberis, Corynebacterium pyogenes, Pseudomonas, Pasteurella, and Brucella abortus, were also detected in mastitis cases.4,6

Antimicrobials have been used frequently as a conventional measure to prevention and control diseases in dairy farming. Especially in mastitis control programs, more and more antibiotics were applied even without any clinical symptoms in dairy cattle herds. However, long term in-feed use of antibiotics on dairy farms has lead to the alarming increase of antibiotic resistant bacteria and which has become a public health issue worldwide, e.g., methicillin-resistant S. aureus (MRSA) from raw milk and environmental samples constitutes a great threat to food safety.7

In order to better understand the potential of dairy cattle as a reservoir for antibiotics resistant bacteria, it is important to investigate the prevalence of antimicrobial resistance among bacteria isolated from raw milk. In this study, Luria-Bertani plates with appropriate antibiotics were used in directly isolating the antibiotics resistant bacteria from raw milk samples. The antimicrobial resistant isolates were identified with 16S rRNA analysis. Their antibiotic resistance phenotypes were further validated with K-B disk diffusion method and their hemolysis types were determined by blood agar plates.

II. MATERIALS

In this study, 60 raw milk samples were collected from Zhangjiakou, Hebei Province. Sampling process was compliance with aseptic procedures strictly. Briefly, washing udder with warm water first, subsequently sterilizing it with 0.2% benzalkonium bromide, then disinfecting teat with 75% alcohol. Hand milking was performed and milk samples were collected in sterile tubes after dumping the first three squirts to clean out the teat. The number and sample source were recorded and samples were stored at 4°C before analyzing.

III. METHODS

A. Preliminary screening of antibiotics resistant bacteria

Milk sample was subjected to 10-fold serial dilutions before spreading on plates. In brief, taking 0.1 mL raw milk and mixing with 0.9 mL sterile phosphate buffered saline samples; subsequently taking 0.1 mL the mixture and mixing with 0.9 mL sterile phosphate buffered saline samples; repeat the step until raw milk was diluted 10⁶. 100 μL mixtures of diluted samples were spread on Luria-Bertani (LB) plates with ampicillin (32 μg/mL), clindamycin (4 μg/mL), or chloramphenicol (32 μg/mL), respectively. The plates were incubated at 35°C for 16 - 18 h and the results were recorded.
B. Isolates identification with 16S rRNA analysis

The structure of 16S rRNA not only has conservative, but also has high variability. The conservative reflects that the genetic relationship of biological species, providing clues for the phylogenetic reconstruction; the high variability that reveals the characteristics of nucleic acid sequence of species is the molecular basis for species identification. The purified chromosome DNA was used as templates in 16S rRNA gene amplifications. PCR was performed as described and specific primers used are as follows:

27F: 5'AGAGTTTGATCCTGCTAG3'
1492R: 5'GGTACCTTGTTACGACTT3'

50μL PCR reaction system:
10×Taq buffer 5 μL
dNTPs 5 μL
27F Primer 2 μL
1492R Primer 2 μL
Template 1 μL
Taq Polymerase 1 μL
ddH2O 3 μL

PCR reaction conditions:
94℃ 5 min
94℃ 40 s
51℃ 2 min 30 cycles
72℃ 3 min
72℃ 15 min
4℃ 2 h

Purified amplification product was subjected to sequencing analysis with the same primers used in PCR. Sequence homology search was carried out with BLAST provided by NCBI to identify the isolates.

C. Hemolysis analysis

Blood agar plates were used in hemolysis analysis. The isolated strains were streaked on the plates and incubated at 35℃ for 24 h. Colony morphology and hemolytic zones around colonies were observed after incubation.

D. Antibiotics resistance validation with K-B disk diffusion method

K-B disk diffusion method was used to test antibiotic resistance phenotypes of the isolates and it was performed according the descriptions. In brief, overnight isolate culture was diluted to suspension of 1.5×10^8 cfu/mL with sterile phosphate buffered saline, and 100 μL was spread on MH agar plate. The disk was attached to the pre-marked position and the diameters of zones of inhibition were measured after 16-18 h incubation at 35℃. Antibiotic susceptibility was determined according to Clinical and Laboratory Standards Institute (CLSI). K-B disks were ampicillin (10 μg), clindamycin (2 μg), and chloramphenicol (30 μg), respectively.

IV. RESULTS

A. Preliminary screening of antibiotic resistant bacteria

Diluted milk samples were spread on LB plates with ampicillin (32 μg/mL), clindamycin (4 μg/mL), or chloramphenicol (32 μg/mL), respectively. Screening results showed that 48 (80%) samples had isolates resistant to more than one antibiotic, 39 (65%) samples had isolates resistant to more than two antibiotics, and 14 (23.33%) samples had isolates resistant to three antibiotics tested. Isolates were counted according their morphology. Totally, 48 isolates were resistant to ampicillin (32 μg/mL), 46 isolates were resistant to clindamycin (4 μg/mL), and 21 isolates were resistant to chloramphenicol (32 μg/mL).

B. Isolates identification with 16S rRNA analysis

To identify the isolates, 10 strains resistant to three antibiotics were randomly selected for 16S rRNA analysis. Genomic DNA was extracted from these strains and used as templates for 16S rRNA gene amplifications. PCR products were purified and sequenced, and DNA sequences were analyzed and compared by BLAST (table I). The searching results were given in table I that eight strains were P aeruginosa (KDZ91, KDZ245, KDZ262, KDZ401, KDZ713, KDZ961, KDZ981, and KDZ992), one strain was S. marcescens (KDZ948), and one strain was E. cloacae (KDZ972).

### TABLE 1: 16S rRNA sequence homology alignments of the isolates

<table>
<thead>
<tr>
<th>Isolates No.</th>
<th>Description</th>
<th>Accession No.</th>
<th>Query coverage</th>
<th>Hemolysis type</th>
</tr>
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<tbody>
<tr>
<td>KDZ91</td>
<td>Pseudomonas aeruginosa</td>
<td>HQ37785.1</td>
<td>100%</td>
<td>α</td>
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<tr>
<td>KDZ245</td>
<td>Pseudomonas aeruginosa</td>
<td>HQ84402.1</td>
<td>99%</td>
<td>γ</td>
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<tr>
<td>KDZ262</td>
<td>Pseudomonas aeruginosa</td>
<td>HQ09587.8</td>
<td>99%</td>
<td>α</td>
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<td>GU124931</td>
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<td>γ</td>
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<tr>
<td>KDZ713</td>
<td>Pseudomonas aeruginosa</td>
<td>GU399616</td>
<td>99%</td>
<td>α</td>
</tr>
<tr>
<td>KDZ961</td>
<td>Pseudomonas aeruginosa</td>
<td>AV149431</td>
<td>99%</td>
<td>γ</td>
</tr>
<tr>
<td>KDZ981</td>
<td>Pseudomonas aeruginosa</td>
<td>JF912978</td>
<td>100%</td>
<td>β</td>
</tr>
<tr>
<td>KDZ992</td>
<td>Pseudomonas aeruginosa</td>
<td>JN051841</td>
<td>100%</td>
<td>γ</td>
</tr>
</tbody>
</table>

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C. Hemolysis analysis of isolates

P. aeruginosa KDZ2401 and KDZ961 formed 1-2 mm grass green rings around the colonies, assigned as α-hemolytic (table II, fig 1); P. aeruginosa KDZ91 and KDZ713 formed a completely clear and transparent hemolytic zone around colonies, assigned as β-hemolytic (table II, fig 1); the left strains had no change around colonies, assigned as γ-hemolytic (table II, fig 1).

D. Antibiotics resistance validation with K-B disk diffusion method

Inhibition zones were measured for each test and the antibiotics susceptibility was determined according to CLSI standards (table II). All isolates were resistant to ampicillin and clindamycin; seven isolates were resistant to chloramphenicol, one isolate intermediate to chloramphenicol, and two isolates sensitive to chloramphenicol.

V. DISCUSSION

In this study, eight of ten randomly selected isolates are P. aeruginosa, one is S. marcescens, and the remaining one is E. cloacae. These strains are all the pathogens causing cattle mastitis. 40% (4/10) of them can secret α or β hemolysis type hemolysins which are virulence factors damaging host mammalian cells.

Antibiotics are widely used in animal feed additives for the prevention of bacterial infections. And it has been raising concerns of public health and food safety issues mainly due to the increasing isolation frequency of antibiotic resistant bacteria. In this study, 41.74% (48/115) strains were isolated resistant to ampicillin, 40% (46/115) strains were isolated resistant to clindamycin, and 18.26% (21/115) strains were isolated resistant to chloramphenicol. These results are consistent with previous studies.

In conclusion, our experiment results show that raw milk is the reservoir of a variety of antibiotics resistant pathogens. Long term unlimited use of antibiotic provides pressures and leads to the enrichments of multidrug resistant bacteria.

REFERENCES


